

Detection of ryanodine receptor antibodies.

The present invention relates to methods, kits and compositions for the detection of ryanodine receptor antibodies in patient serum samples. The invention also relates to a method for the manufacture of a pharmaceutical agent for the prevention and/or treatment of the disease myasthenia gravis, and a method of myasthenia gravis prognosis.

Myasthenia gravis (MG) is a disease of the neuromuscular junction caused and characterised by antibodies against the Acetyl Choline Receptor (AChR) of the muscle endplate. The AChR antibodies cause a complement mediated damage to the postsynaptic part of the muscle endplate leading to impaired neuromuscular transmission, muscular weakness and fatiguability (Lindstrom, J., D. Schelton, and Y. Fujii. 1988, *Adv. Immunol.* 42: 233-284). However, some MG patients, mainly with thymoma, have antibodies also against other muscle antigens.

Electron microscopic studies have shown that MG sera bind to an unidentified protein in sarcoplasmic reticulum (SR) membranes (Mendell, J.R., J.N. Whitaker, and W.K. Engel. 1973, *J. Immunol.* 111: 847-856; Flood, P.R., R. Bjugn, N.E. Gilhus, H. Hofstad, R. Matre, and J.A. Aarli. 1987, *Ann. N.Y. Acad. Sci.* 505: 732-734). Mygland et al., showed that MG sera from thymoma patients stained a high molecular weight protein in a SR preparation (Mygland, Å., O.B. Tysnes, J.A. Aarli, P.R. Flood, and N.E. Gilhus. 1992, *J. Neuroimmunol.* 37: 1-7), which was later identified to be

identical to the Ca^{2+} release channel of SR, i.e. the ryanodine receptor (RyR) (Mygland, Å., O.B. Tysnes, R. Matre, P. Volpe, J.A. Aarli, and N.E. Gilhus. 1992, *Ann. Neurol.* 32: 589-591).

5 The RyR is a Ca^{2+} -release channel located in the SR of striated muscle. It plays an essential role in muscle contraction by responding to sarcolemma depolarisation with the opening of the ion channel and the release of Ca^{2+} from SR to contractile proteins in the myoplasm (Coronado, R.,
10 J. Morrisette, M. Sukhareva, and D.M. Vaughan. 1994, *Am. J. Physiol.* 266: c1485-c1504).

RyR antibodies were found in approximately half of thymoma MG patients but not in non-thymoma late-onset MG, early onset MG, blood-donors or patients with other
15 autoimmune diseases (Mygland, Å., O.B. Tysnes, R. Matre, P. Volpe, J.A. Aarli, and N.E. Gilhus. 1992, *Ann. Neurol.* 32: 589-591). The RyR antibodies are mainly of the IgG1 and IgG3 subclasses (Mygland, Å., O.B. Tysnes, J.A. Aarli, R. Matre, and N.E. Gilhus. 1993, *J. Autoimmunity.* 6: 507-515),
20 and stain both the skeletal and cardiac form of the RyR (Mygland, Å., O.B. Tysnes, R. Matre, J.A. Aarli, and N.E. Gilhus. 1994, *Autoimmunity* 17: (4) 327-31).

The presence of RyR antibodies correlate to MG severity and even death with a nice correlation also to
25 RyR-antibody levels. Thymoma MG patients with RyR antibodies have in contrast to RyR negative patients often heart disease leading to sudden cardiac arrest. Others die of respiratory failure due to the neuromuscular weakness (Mygland, Å., O.B. Tysnes, R. Matre, J.A. Aarli, and N.E. Gilhus. 1994, *Autoimmunity* 17: (4) 327-31.; Mygland, Å., J.A. Aarli, R. Matre, and N.E. Gilhus. 1994, *J. Neurol. Neurosurg. Psychiatry.* 57: 843-846; Skeie, G.O., E. Bartoccioni, A. Evoli, J.A. Aarli, and N.E. Gilhus. 1996, *Eur. J. Neurol.* 3: 136-140).

35 The mechanisms leading to muscular fatiguability in MG patients might be more complex than what can be explained

from the AChR antibody model alone. Pagala and others have found evidence for disordered (Pagala, M., N.V. Nandakumar, S.A.T. Venkatachari, K. Ravindran, T. Namba, and D. Grob. 1990, *Muscle and Nerve* 13: 1012-1022; Pagala, M., N.V.

- 5 Nandakumar, S.A.T. Venkatachari, K. Ravindran, B. Amaladevi, T. Namba, and D. Grob. 1993, *Muscle and Nerve* 16: 911-921) excitation-contraction coupling, for which the RyR is essential, in individual MG patients. The MG patients RyR antibodies are able to inhibit binding of
10 ryanodine to the RyR indicating that the RyR antibodies lock the RyR in the closed position (Skeie, G.O., P.K. Lunde, O.M. Sejersted, Å. Mygland, J.A. Aarli, and N.E. Gilhus. 1998, *Muscle and Nerve* 21: 329-33). Patients with inhibiting antibodies had a more severe disease than
15 patients without such antibodies (Skeie et al., 1998).

MG sera containing striational antibodies have been shown to inhibit caffeine induced Ca^{2+} release in rat muscle cells (Asako et al., 1997). Experimental RyR antibodies can affect RyR function in vitro (Treves S,
20 Chiozzi P, Zorzato F (1993) *Biochem J* 291, 757-763) and a rat strain which develop spontaneous thymomas and RyR antibodies have muscular weakness and fatiguability resembling MG without detectable AChR antibodies (Iwasa, K., K. Komai, T. Asaka, E. Nitta, and M. Takamori, *Ann.*
25 *N.Y. Acad. Sci.* 1998:841; 542-545). These studies might indicate a direct pathogenetic role for the RyR antibodies in MG, and thus RyR antibodies do not only have a function as a disease marker.

In this study we have identified the main immunogenic
30 region on the RyR for MG patients antibodies. We show that the antibodies reactive with this part of the RyR are able to inhibit Ca^{2+} release from SR vesicles in vitro, and using a biosensor we were able to study the real time interaction between the RyR antibodies and the RyR fusion
35 protein containing the MIR for the RyR antibodies in MG sera.

The present invention relates to a method for the detection of ryanodine receptor antibodies in patient serum samples, said antibodies being associated with the disease myasthenia gravis, said method comprising the following steps;

(a) obtaining a serum sample from a patient suspected of having myasthenia gravis or being at risk for the development of said disease;

(b) contacting said serum sample with a composition of fusion proteins comprising the following sequences: SEQ ID NO 1 or SEQ ID NO 2;

c) detecting fusion protein - antibody complex formation, wherein said detected complexes indicate the presence of ryanodine receptor antibodies.

Further, the invention relates to the use of the fusion proteins comprising the sequences SEQ ID NO 1 or SEQ ID NO 2 for the detection of RyR antibodies.

Also comprised by the invention is a diagnostic kit for the detection of ryanodine receptor antibodies in patient serum samples, said antibodies being associated with the disease myasthenia gravis, said kit comprising fusion proteins having the following sequences; SEQ ID NO 1 or SEQ ID NO 2.

A preferred embodiment of the invention relates to a diagnostic kit, wherein the immunodetection reagent is a radiolabelled reagent.

The presence of pc2 or pc25 fusion protein antibodies is indicative of the presence of a thymoma.

The present invention also relates to a composition of fusion proteins useful for the detection of ryanodine receptor antibodies, which are associated with the disease myasthenia gravis, said proteins being selected from the group of proteins having a sequence of SEQ ID NO 1 or SEQ ID NO 2, or a combination of said sequences, and to a method for the manufacture of a pharmaceutical agent for the prevention and/or treatment of the disease myasthenia

gravis, wherein said agent are administered to a patient in need thereof, in a amount sufficient to inhibit the binding of ryanodine receptor antibodies to the ryanodine receptor, said composition comprising a panel of fusion proteins

5 having sequences SEQ ID NO 1 and/or SEQ ID NO 2, and to a method of myasthenia gravis prognosis which involves the determination of the presence of RyR antibodies wherein the RyR antibodies are identified by the use of the fusion proteins pc2 and pc25.

10 The invention will now be further described with respect to the following examples and the accompanying figures, in which:

Figure 1A shows in diagrammatic form the Ryanodine receptor fusion protein strategy. Figure 1B shows the
15 induced *E. coli* extracts loaded in the gel. Proteins were separated by electrophoreses, blotted into nitrocellulose and stained with Ponceau Red. The lower row shows the Western blot staining.

Figure 2A shows a Ryanodine receptor fusion protein
20 strategy designed to narrow the immunopositive region. Figure 2B shows the induced *E. coli* extracts loaded in the gel. The lower row shows the Western blot staining (stained with Ab from patients).

Figure 3 shows the fragments pc2, pc2A and pc2B
25 blotted onto nitrocellulose membranes and stained with Ab from patient

Figure 4 shows the characterisation of the binding between MG patients' antibodies and the pc2 fusion protein. Figure 4A shows the curves from injections with anti-path
30 antibody in concentrations ranging from 2.5-25 mg/ml (Puickinject, flowrate 5 µl/min in HBS, pH 7.4) in the flowcell with bound pc2. The maximal response was 400 RU.

Figure 4B shows the results from injections of IgG fractions from MG patients and controls. Non-covalently
35 bound proteins were removed and the sensor ship regenerated by injections of 5 µl of 0.5 % SDS and/or 3 M guanidinium

chloride in 5 mM Tris, pH 8, between injections of serum or IgG samples.

Figure 4C shows the results from the sandwich assay. The anti-path antibody was immobilised on the sensor chip, and a) the injection of electroeluted pc2 Ry1 fusion protein gave a signal of 60 RU, and b) the second injection of Ry1 antibody positive MG IgG lead to an additional increase of 180 RU which is consistent with a Ab binding stoichiometry of 1.

Figure 5 shows 4-chloro-m-cresol (4-cmc) induced Ca^{2+} release from skeletal muscle SR. Ca^{2+} concentrations are represented by the A710-790 of the Ca^{2+} indicator antipyrilazo III. SR was added to the cuvette followed by 6 consecutive additions of 20 nmol CaCl_2 to load the vesicles with Ca^{2+} . 50 ml of IgG (0.5 mg/ml) from patients and controls were added to the cuvettes for 2 minutes incubation, followed by addition of 4-chloro-m-cresol (200 mM) to induce Ca^{2+} release. Curve A shows a normal Ca^{2+} release with IgG from a RyR negative MG patient. Curve B shows that IgG from a Ry1 positive patient strongly inhibits Ca^{2+} release. Curve C shows a normal Ca^{2+} release after removing Ry1 antibodies from the IgG by preincubation with pc2.

Figure 6 shows the 4-chloro-m-cresol induced Ca^{2+} release from SR reacted with IgG from MG patients and controls.

Figure 7 shows the Ca^{2+} release from SR reacted with IgG fractions with and without Ry1 antibodies at different 4-Chloro-m-cresol concentrations. The Ry1 antibodies shift the curve to the right suggesting an allosteric inhibition.

Experimental section

Methods and results

The sera used in the assay

The study included sera from 122 (75) thymoma MG patients (37 Italian, 38 Norwegian, 19 late-onset MG patients and 25 early-onset MG patients (all Norwegian) which had previously been tested in WB for RyR antibodies using a SR preparation as antigen (Mygland et al., 1992; Skeie, G.O., E. Bartoccioni, A. Evoli, J.A. Aarli, and N.E. Gilhus. 1996, *Eur. J. Neurol.* 3: 136-140). The medical records were reviewed and the patients scored according to MG severity at peak of illness and at the last follow-up as previously described (Mygland et al., 1994; Skeie et al., 1996; Skeie, G.O., Å. Mygland, J.A. Aarli, and N.E. Gilhus. 1995, *Autoimmunity* 20: 99-105). In addition sera from 20 Norwegian blood-donors and 3 SLE patients were used as controls.

Biosensor and calcium release

For the biosensor and calcium release assay purified IgG fractions from patient and control sera were obtained using protein G columns according to the protocols provided by Pharmacia AB (Uppsala, Sweden). The IgG fractions were dialysed against HBS buffer and the concentrations adjusted before use to 0.5 mg/ml.

Screening of overlapping RyR fusion proteins

DNA manipulations were carried out as described in Maniatis P, Fritsch EF, Sambrook J. *Molecular Cloning*. A panel of fusion proteins covering the entire RyR coding sequence were constructed as previously described (Treves S, Chiozzi P, Zorzato F (1993) *Biochem J* 291, 757-763; Menegazzi P, Larini F, Treves S, Guerrini R, Quadroni M, Zorzato F, (1994) *Biochemistry* 33, 9078-9084). Gel electrophoresis was carried out as described by Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685. Western blots of bacterial extracts were carried out overnight as described by Gershoni et al., (1985). Indirect

immunoenzymatic staining of Western blots was carried out as described by Young, R.A., B.R. Bloom, C.M. Grosskinsky, J. Ivanyi, D. Thomas, and R.W. Davis. 1985, *Proc. Natl. Acad. Sci. U. S. A.* 82:2583-2587), and detailed by Treves et al., (Treves S, Chiozzi P, Zorzato F (1993) *Biochem J* 291, 757-763).

RyR fusion protein Western blot

We used the pc2-RyR fusion protein as antigen (Mygland, 1992). Electrophoresis was performed on sodium dodecyl sulphate (SDS) polyacrylamide gels (12%) as described by Laemmli, 1970. 200 ml pc2 (50 mg/ml) were added to 120 ml of sample buffer containing 2% (w/v) SDS, 1.5% (w/v) Tris, 10% (v/v) glycerol and 0.001% (w/v) bromophenol blue. The mixture was heated to 100°C for 1 minute. About 100 µl of the protein mixture was applied per gel. Proteins separated on the gel were transblotted onto nitrocellulose sheets as described by Towbin, H., T. Staelin, and J. Gordon. 1979, *Proc. Natl. Acad. Sci. U.S.A.* 76: 4350-4354.

Nitrocellulose sheets were soaked in 5% (w/v) low fat dry milk (Nestle) in phosphate buffered saline (PBS) for 1 hour to block additional protein binding sites. They were washed 3 times in PBS with 0.05% Tween 20 (PBS-Tween), cut into vertical strips and incubated over night at 4 °C with patient and control sera diluted 1:50 in PBS containing 0.5% fat free dry milk and 0.05% Tween 20 (PBS-dry milk Tween (Nestle)). After separate washings for 10 min in PBS-Tween, the nitrocellulose strips were incubated for 1 h with peroxidase-conjugated rabbit antibodies (Dako, Copenhagen, Denmark) to human IgG diluted 1:1000 in PBS dry milk Tween. The nitrocellulose strips were then washed and developed in a peroxidase colour development solution containing 30 mg 4-chloro-1-naphthol (Sigma), 17% (v/v) cold methanol, 83% (v/v) PBS and 0.05% (v/v) H₂O₂. Positive

and negative control sera were applied to strips from each transblotted nitrocellulose sheet.

Results

5 Example 1

The identification of the main immunogenic region of RyR

As shown in figures 1 and 2, MG sera reacted with two of the RyR fusion proteins examined. The reactivity was
10 strongest with the pc2 epitope which is located near the N-terminus. The pc2 epitope comprises residues 799-1152 and consists of 360 amino acids. The pc25 epitope is located between residues 2595-2935. The amino acid sequences (one-letter codes) of the pc2 (SEQ ID NO 1) and pc25 (SEQ ID NO
15 2) epitopes are given below.

When the pc2 peptide was clipped into 2 fragments (indicated as pc2A and pc2B in figure 3) by Pst I the reactivity with the MG sera was lost indicating that the site for the restriction enzyme is located very near the
20 binding site for the protein, or that this site is important for the conformation recognised by the MG patients antibodies.

Antibodies against pc2 were found in 57 out of 75 thymoma MG patients, 5 out of 19 late-onset MG patients,
25 none of 25 MG hyperplasia patients and none of the 20 blood-donors.

24 of the 44 thymoma MG patients examined for antibodies against pc25 in WB had antibodies reactive with this RyR epitope, but none of 20 MG hyperplasia patients
30 and none of the 20 blood-donors. All patients positive for pc25 had pc2 antibodies while only 24 of the 33 pc2 positive patients had pc25 antibodies.

All sera with reactivity against the full length RyR in Western blots did also react with the pc2 RyR fusion
35 protein (Table 1). The pc2 RyR fusion protein must therefore contain the main immunogenic region.

TABLE 1:

- 5 Number of MG patients with Ry1 antibodies using different
 Ry1 antigens in WB.

	Thymoma MG	Late-onset MG	Early onset MG
Ry1 antibodies 44/75 (SR)		0/19	0/25
Ry1 antibodies 57/75 (pc2)		5/19	0/25
Ry1 antibodies 24/44 (pc25)		nd	0/25

SR: sarcoplasmic reticulum, pc2: pc2 Ry1 fusion protein,
 pc25: pc25 Ry1 fusion protein.

10

Correlation with titin antibodies

All but 9 thymoma MG patients had titin antibodies. 13
 titin positive MG patients had no pc2 RyR antibodies, while
 4 patients with pc2 RyR antibodies had no titin antibodies.
 5 thymoma MG patients were negative for both titin and pc2
 RyR antibodies. 10 out of the 19 late onset sera contained
 titin antibodies. 5 of the sera also contained MG anti-pc2
 antibodies while 5 sera with titin antibodies had no pc2
 RyR antibodies.

20

We established an ELISA (data not shown) using the pc2
 fusion protein as antigen. The results must be interpreted
 together with the Western blot data since some sera
 negative with the pc2 band in WB had a little background
 staining of residual bacterial proteins that gave a low
 positive ELISA signal. The ELISA could therefore be used as
 a screening test before checking all positive sera for
 reactivity with the pc2 RyR fusion protein in WB to
 increase the specificity.

25

Example 2Real-time RyR antibody pc2 fusion protein interactions

The reactivity of MG sera with the pc2 fusion protein was studied using a biosensor; BIACORE 1000 (Pharmacia Biosensor AB, Uppsala, Sweden) that allows real time biospecific interaction analysis. This system uses the optical phenomenon of surface plasmon resonance (SPR) that detects changes in optical properties at the surface of a thin gold film on a glass support (sensor ship) (Lofas and Johnsson, 1990). The sensorship is covered by a dextran matrix to which one reactant is covalently linked, while the other(s) is introduced in a flow passing over the surface. The resonance angle depends on the refractive index in the vicinity of the surface, which changes as the concentration of molecules on the surface is modified and is expressed in resonance units (RU). A signal of 1000 RU corresponds approximately to a surface concentration change of 1 ng/mm².

Immobilisation of protein to the sensor ship were done via primary amine groups using the amine coupling kit (Pharmacia Biosensor AB) according to standard procedures (Lofas and Johnsson 1990; Fagerstam LG, Frostell A, Karlsson R, Kullman M, Larsson A, Malmqvist M and Butt H. (1990), J Mol Recog. 3, 208-214). The carboxylated matrix of the sensorship CM5 (Pharmacia Biosensor AB) was first activated by injection (Quickinject, flow rate 5 ml/min in HBS pH 7.4 (10 Hepes, 150 mM NaCl, 3.4 mM EDTA, 0.05% Surfactant P20 (Pharmacia Biosensor AB) of a mixture of NHS/EDS (N-hydroxy-succinimide 50 mM/N-ethyl-N-(3 diethylaminopropyl)-carbodiimide 200 mM) (50 ml). Then in one flowcell 60 ml pc2 RyR fusion protein (1.5 mg/ml in 2.5 mM acetate buffer, pH 4) was shown to give the best binding in preconcentration experiments using buffers of different pH. About 2000 RU were immobilised on the sensorship.

In another flowcell about 4000 RU of an anti-path antibody were immobilised by injecting 70 ml of anti-path antibody (35 ml anti-path 0.5 mg/ml mixed with 35 ml 10 mM acetate buffer, pH 5.5). Residual activated sites were
5 blocked by injection of 50 ml of 1 M ethanolamine hydrochloride pH 8.5 (Pharmacia Biosensor AB).

In one flow cell the sensorship was only activated by NHS/EDS and blocked by ethanolamine hydrochloride without injection of any proteins. This flow cell was used to
10 examine non-specific IgG binding to the dextrane matrix of the sensorship. 50 ml of sera diluted 1:10-1:100 in HBS buffer and purified IgG fractions (dialysed against HBS buffer) (0.5 mg/ml) from MG patients and controls were injected into the flow cells at a constant flow rate of 5
15 ml/ml and a sensorgram recorded. Between injections the sensor chip was continuously washed with HBS buffer.

Further, different concentrations of anti-path antibody were injected into the flow cell with immobilised pc2 fusion protein to examine the amount and reactivity of
20 the immobilised pc2 protein, and to compare the binding kinetics with the MG sera.

In the flow cell with immobilised anti-path antibodies 50 ml of pc2 fusion protein were injected prior to the injection of sera/IgG fractions. The sensor ship were
25 regenerated by injections of 5 ml of 5% SDS and/or 3 M guanidinium chloride in 5 mM Tris pH 8 between injections of serum or IgG samples.

The curves from sensorgrams obtained by injecting the samples into the flow cell without proteins were subtracted
30 from the curves obtained in flow cells with immobilised pc2 RyR fusion protein to record the specific binding.

Real time Surface Plasmon Resonance Recording

About 2000 RU of the pc2 fusion protein was
35 immobilised on the sensorship. When injecting different concentrations of the high affinity anti-path antibody a

maximal response of 400 RU was obtained. This was therefore the maximal expected response also for the patient sera. The results from injections of 50 ml of purified IgG fractions 0.5 mg/ml from MG patients and controls are shown in Figure 4B. Only IgG fractions from RyR antibody positive MG patients gave a signal above 30 RU (73-360 RU). The sera with the best binding gave a signal close to the maximal expected, indicating that nearly all binding sites for the RyR antibodies were saturated.

The specificity of the interaction was also tested in a sandwich assay. When injecting 50 ml (1 mg/ml) of pc2 RyR fusion protein into the flow cell with immobilised anti-path antibody, 60 RU were bound, as shown in Figure 4C. The second injection of 50 ml RyR antibody positive MG IgG (0.5 mg/ml) gave a signal of about 180 RU (Figure 4C).

The molecular weight of pc2 is 60kD, and the molecular weight of the antibodies is 150 kD. As there is a 1:3 relationship between both the MW and signal in RU there is a 1:1 relationship between the RyR antibodies and the fusion protein i.e. one antibody molecule binds to one RyR fusion protein.

Example 3.

Inhibition of Ca^{2+} release

Ca^{2+} measurements

SR was isolated from white muscles of New Zealand White rabbits and was fractionated into longitudinal tubules and TC in the presence of antiproteolytic agents as described by Saito, A., S. Seiler, A. Chu, and S. Fleischer. 1984, *J. Cell. Biol.* 99:875-885. The SR fractions were resuspended in 0.3 M sucrose, 5 mM imidazole, pH 7.4, 100 mM PMSF, 1 mg/ml leupeptin, and were stored in liquid nitrogen until used.

Ca^{2+} release from isolated SR fractions was measured in a Beckmann DU7400 diode array spectrophotometer by

monitoring the $A_{710}-A_{790}$ value of the Ca^{2+} indicator antipyrilazo III (Fluca, Buchs, Switzerland) as described by Palade, P. 1987, *J Biol. Chem.* 262: 6142-6148, and detailed by Treves S, Chiozzi P, Zorzato F (1993), *Biochem J* 291, 757-763. Pulses of 20 nM Ca^{2+} were administered to load the SR fractions with Ca^{2+} , and the fractions were then incubated with 50 ml of IgG (0.5 mg/ml) from patients and controls for 2 minutes before different concentrations of the Ca^{2+} releasing agent 4-chloro-m-cresol were added. To calibrate the curves 20 nM of Ca^{2+} were added at the end of each experiment.

IgG fractions from patients with RyR antibodies significantly inhibited 4-chloro-m-cresol induced Ca^{2+} release from isolated SR vesicles (Figure 5). The mean Ca^{2+} release rate was significantly lower when the SR vesicles had been incubated with IgG fractions from RyR antibodies positive MG patients (0.93 ± 0.55 mmol Ca^{2+} per mg SR protein per min) compared with IgG fractions from RyR antibody negative MG patients (1.6 ± 0.36 mmol Ca^{2+} per mg SR protein per min) and controls (blood-donors and SLE patients) (1.6 ± 0.21 mmol Ca^{2+} per mg SR protein per min) ($p=0.0021$). The inhibition was concentration dependent (Figure 7) and the curves fitted with a model of allosteric inhibition.

When removing the RyR antibodies by preabsorbing the IgG fractions with the pc2 fusion protein or the SR fractions for 1 hour before using them in the Ca^{2+} release assays the inhibition of Ca^{2+} release disappeared (Figure 5), indicating that the antibodies binding to the pc2 RyR fusion protein are responsible for inhibiting Ca^{2+} release *in vitro*.

Conclusions

By mapping a series of overlapping RyR fusion proteins we have identified the main immunogenic region (MIR) on the RyR for MG patients antibodies. All patients positive for

RyR antibodies in Western blots using a SR preparation as antigen had pc2 antibodies proving that the pc2 fusion protein contains the MIR region. However, 13 patients positive for MIR antibodies were negative in the SR Western blot assay, indicating that the use of the recombinant protein as antigen gives a more sensitive assay. The MIR RyR antibodies were found in 76% of thymoma MG patients but not in blood-donors or young-onset MG patients. The antibodies are therefore closely associated with thymoma MG and the presence of the pc2 fusion protein antibodies strongly suggests the presence of a thymoma. The only patients without a detectable thymoma, positive for the pc2 RyR antibodies, were 5 late-onset MG patients. All of these also had titin antibodies. The late onset MG patients with titin/RyR antibodies are very similar to thymoma MG patients immunologically, clinically and genetically (Aarli, J.A. 1997. Late-onset MG. *Eur. J. Neurol.* 4: 203-209). Microscopic thymomas has been described by Pescarmona, E., S. Rosati, A. Pisacane, E.A. Rendina, F. Venuta, C.D. Baroni. 1992. *Histopathology*. 20: 263-266), and it is not unlikely that the late-onset MG patients with titin and RyR antibodies have a paraneoplastic MG much like the thymoma patients. Some could have a preneoplastic condition; paraneoplastic symptoms often develop years before a tumour is found in a great proportion of patients with other paraneoplastic conditions (Dropcho, E.J. 1998. *Ann. N.Y. Acad. Sci.*: 841:246-261), or the thymoma could have gone into remission in a way similar to that described for other tumours (Dropcho, 1998). RyR antibody positive MG patients should probably be thymectomized regardless of a positive CT scan. The results from the surface plasmon resonance studies showed that there is a strong, specific one-to-one interaction between the MG patients' antibodies and the pc2 RyR fusion protein. The exact binding kinetics of the interaction could not be measured, as the exact concentration of the polyclonal RyR antibodies in the IgG

fractions was unknown. However, using different concentrations of IgG from different patients in curve fitting models where the RyR antibody concentration was set to be 1-10% of the total IgG concentrations, K_D was estimated to be about 10^{-9} (data not shown). Since the Western blot conditions are also for high affinity antibodies, we conclude that the pc2 RyR antibodies are of high affinity.

The pc2 RyR antibodies inhibited cresol activated Ca^{2+} release from isolated SR fractions in a concentration dependant manner suggesting allosteric inhibition. We have previously shown that MG sera containing RyR antibodies inhibit binding of ryanodine to the RyR, which also indicate that the RyR antibodies lock the RyR in the closed position. The RyR antibodies reacting with pc2 were responsible for this effect since the inhibition of Ca^{2+} release disappeared when the pc2 antibodies were removed from the IgG fractions. The pc2 region is located near a clipping site for a protease and therefore probably located on the surface of the cytoplasmic part and the RyR, the foot-region. A portion of this region of the molecule is also interacting with another region on the neighbouring region of the RyR tetramer, and is therefore probably important for the conformation of the receptor (Wu, Y., B. Aghdasi, S.J. Dou, J.Z. Zhang, S.Q. Liu, and S.L. Hamilton. 1997, *J. Biol. Chem.* 272: 25051-25061). A potential calmodulin binding site is also located near the pc2 region. Calmodulin is very important for RyR regulation. The MG patients' RyR antibodies do probably interfere with RyR function; locking the receptor in the closed position; by affecting calmodulin binding or interfering with the "self association between the subunits". By mapping the exact epitope for the MG RyR antibodies one might learn more about RyR function.

Whether antibodies against intracellular molecules are of any pathogenic significance is controversial (Alarcon-

Segovia, D., A. Ruiz-Arguelles, and L. Llorente. 1996, *Immunol. Today*. 17:163-164). Antibodies are able to penetrate the cell membrane and can often be found intracellularly bound to their target antigens (not shown for the RyR antibodies). How they get there and whether they can exert their effector functions is unknown. However, this study shows that the RyR antibodies do affect RyR function directly so if they could also penetrate the cell membrane one would expect a severe effect on muscle function as the antibodies bind to the receptor with high affinity and seem to lock the channel in the closed state thus inhibiting Ca^{2+} release and muscle contraction.

SEQUENCE LISTING

5 GENERAL INFORMATION:

- (110) APPLICANT: UNIFOB, Stiftelsen
Universitetsforskning Bergen,
Prof. Keyserstgt. 8
10 5007 Bergen
- (120) TITLE OF THE INVENTION: Detection of ryanodine
receptor antibodies.
- 15 (130) FILE REFERENCE:
(A) Medium type: Diskette
(B) Computer: IBM compatible
(C) Operating system: Windows 98
(D) Software: Word 6.0
- 20 (150) Earlier patent application:
(A) Application number: NO 19992786
(B) Filing date: 08-JUN-1999
- 25 (160) NUMBER OF SEQUENCE ID NOS: 2

(210) INFORMATION FOR SEQ ID NO: 1

(i) Sequence characteristics:

5 (A) Length: 374 amino acid residues
 (B) Type: amino acid
 (D) Topology: single

(xi) Sequence description: SEQ ID NO: 1

10

	Glu	Phe	Lys	Phe	Leu	Pro	Pro	Pro	Gly	Tyr	
1					5					10	
	Ala	Pro	Cys	His	Glu	Ala	Val	Leu	Pro	Arg	
					15					20	
15	Glu	Arg	Leu	Arg	Leu	Glu	Pro	Ile	Lys	Glu	
					25					30	
	Tyr	Arg	Arg	Glu	Gly	Pro	Arg	Gly	Pro	His	
					35					40	
	Leu	Val	Gly	Pro	Ser	Arg	Cys	Leu	Ser	His	
20					45					50	
	Thr	Asp	Phe	Val	Pro	Cys	Pro	Val	Asp	Thr	
					55					60	
	Val	Gln	Ile	Val	Leu	Pro	Pro	His	Leu	Glu	
					65					70	
25	Arg	Ile	Arg	Glu	Lys	Leu	Ala	Glu	Asn	Ile	
					75					80	
	His	Glu	Leu	Trp	Ala	Leu	Thr	Arg	Ile	Glu	
					85					90	
	Gln	Gly	Trp	Thr	Tyr	Gly	Pro	Val	Arg	Asp	
30					95					100	
	Asp	Asn	Lys	Arg	Leu	His	Pro	Cys	Leu	Val	
					105					110	
	Asn	Phe	His	Ser	Leu	Pro	Glu	Pro	Glu	Arg	
					115					120	
35	Asn	Tyr	Asn	Leu	Gln	Met	Ser	Gly	Glu	Thr	
					125					130	
	Leu	Lys	Thr	Leu	Leu	Ala	Leu	Gly	Cys	His	
					135					140	
	Val	Gly	Met	Ala	Asp	Glu	Lys	Ala	Glu	Asp	
40					145					150	
	Asn	Leu	Lys	Lys	Thr	Lys	Leu	Pro	Lys	Thr	
					155					160	
	Tyr	Met	Met	Ser	Asn	Gly	Tyr	Lys	Pro	Ala	
					165					170	
45	Pro	Leu	Asp	Leu	Ser	His	Val	Arg	Leu	Thr	
					175					180	
	Pro	Ala	Gln	Thr	Thr	Leu	Val	Asp	Arg	Leu	
					185					190	
	Ala	Glu	Asn	Gly	His	Asn	Val	Trp	Ala	Arg	
50					195					200	

	Asp	Arg	Val	Ala	Gln	Gly	Trp	Ser	Tyr	Ser	
					205					210	
	Ala	Val	Gln	Asp	Ile	Pro	Ala	Arg	Arg	Asn	
					215					220	
5	Pro	Arg	Leu	Val	Pro	Tyr	Arg	Leu	Leu	Asp	
					225					230	
	Glu	Ala	Thr	Lys	Arg	Ser	Asn	Arg	Asp	Ser	
					235					240	
10	Leu	Cys	Gln	Ala	Val	Arg	Thr	Leu	Leu	Gly	
					245					250	
	Tyr	Gly	Tyr	Asn	Ile	Glu	Pro	Pro	Asp	Gln	
					255					260	
	Glu	Pro	Ser	Gln	Val	Glu	Asn	Gln	Ser	Arg	
					265					270	
15	Trp	Asp	Arg	Val	Arg	Ile	Phe	Arg	Ala	Glu	
					275					280	
	Lys	Ser	Tyr	Thr	Val	Gln	Ser	Gly	Arg	Trp	
					285					290	
20	Tyr	Phe	Glu	Phe	Glu	Ala	Val	Thr	Thr	Gly	
					295					300	
	Glu	Met	Arg	Val	Gly	Trp	Ala	Arg	Pro	Glu	
					305					310	
	Leu	Arg	Pro	Asp	Val	Glu	Leu	Gly	Ala	Asp	
					315					320	
25	Glu	Leu	Ala	Tyr	Val	Phe	Asn	Gly	His	Arg	
					325					330	
	Gly	Gln	Arg	Trp	His	Leu	Gly	Ser	Glu	Pro	
					335					340	
30	Phe	Gly	Arg	Pro	Trp	Gln	Ser	Gly	Asp	Val	
					345					350	
	Val	Gly	Cys	Met	Ile	Asp	Leu	Thr	Glu	Asn	
					355					360	
	Thr	Ile	Ile	Phe	Thr	Leu	Asn	Gly	Glu	Val	
					365					370	
35	Leu	Met	Ser	Asp							
					374						

(210) INFORMATION FOR SEQ ID NO: 2

(i) Sequence characteristics:

5 (A) Length: 348 amino acid residues
 (B) Type: amino acid
 (D) Topology: single

(xi) Sequence description: SEQ ID NO: 2

10 Arg Gly Arg Ser Leu Thr Lys Ala Gln Arg
 1 5 10
 Asp Val Ile Glu Asp Cys Leu Met Ala Leu
 15 15 20
 Cys Arg Tyr Ile Arg Pro Ser Met Leu Gln
 25 25 30
 His Leu Leu Arg Arg Leu Val Phe Asp Val
 35 35 40
 Pro Ile Leu Asn Glu Phe Ala Lys Met Pro
 20 45 50
 Leu Lys Leu Leu Thr Asn His Tyr Glu Arg
 55 60
 Cys Trp Lys Tyr Tyr Cys Leu Pro Thr Gly
 65 70
 25 Trp Ala Asn Phe Gly Val Thr Ser Glu Glu
 75 80
 Glu Leu His Leu Thr Arg Lys Leu Phe Trp
 85 90
 Gly Ile Phe Asp Ser Leu Ala His Lys Lys
 30 95 100
 Tyr Asp Gln Glu Leu Tyr Arg Met Ala Met
 105 110
 Pro Cys Leu Cys Ala Ile Ala Gly Ala Leu
 115 120
 35 Pro Pro Asp Tyr Val Asp Ala Ser Tyr Ser
 125 130
 Ser Lys Ala Glu Lys Lys Ala Thr Val Asp
 135 140
 Ala Glu Gly Asn Phe Asp Pro Arg Pro Val
 40 145 150
 Glu Thr Leu Asn Val Ile Ile Pro Glu Lys
 155 160
 Leu Asp Ser Phe Ile Asn Lys Phe Ala Glu
 165 170
 45 Tyr Thr His Glu Lys Trp Ala Phe Asp Lys
 175 180
 Ile Gln Asn Asn Trp Ser Tyr Gly Glu Asn
 185 190
 Val Asp Glu Glu Leu Lys Thr His Pro Met
 50 195 200

	Leu	Arg	Pro	Tyr	Lys	Thr	Phe	Ser	Glu	Lys	
					205					210	
	Asp	Lys	Glu	Ile	Tyr	Arg	Trp	Pro	Ile	Lys	
					215					220	
5	Glu	Ser	Leu	Lys	Ala	Met	Ile	Ala	Trp	Glu	
					225					230	
	Trp	Thr	Ile	Glu	Lys	Ala	Arg	Glu	Gly	Glu	
					235					240	
	Glu	Glu	Arg	Thr	Glu	Lys	Lys	Lys	Thr	Arg	
10					245					250	
	Lys	Ile	Ser	Gln	Thr	Ala	Gln	Thr	Tyr	Asp	
					255					260	
	Pro	Arg	Glu	Gly	Tyr	Asn	Pro	Gln	Pro	Pro	
					265					270	
15	Asp	Leu	Ser	Gly	Val	Thr	Leu	Ser	Arg	Glu	
					275					280	
	Leu	Gln	Ala	Met	Ala	Glu	Gln	Leu	Ala	Glu	
					285					290	
	Asn	Tyr	His	Asn	Thr	Trp	Gly	Arg	Lys	Lys	
20					295					300	
	Lys	Gln	Glu	Leu	Glu	Ala	Lys	Gly	Gly	Gly	
					305					310	
	Thr	His	Pro	Leu	Leu	Val	Pro	Tyr	Asp	Thr	
					315					320	
25	Leu	Thr	Ala	Lys	Glu	Lys	Ala	Arg	Asp	Arg	
					325					330	
	Glu	Lys	Ala	Gln	Glu	Leu	Leu	Lys	Phe	Leu	
					335					340	
	Gln	Met	Asn	Gly	Tyr	Ala	Val	Thr			
30					345					348	